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The threat of biological warfare and bioterrorism has increased in the last two decades. Classical biological threats that were part of the now-defunct U.S. offensive program (terminated in 1969) included Bacillus anthracis, Botulinum toxin, Francisella tularensis, Coxiella burnetii, Venezuelan equine encephalitis (VEE), Brucella suis(brucellosis), Staphylococcal enterotoxin B (SEB), rice blast, rye stem rust, and wheat stem rust. Most of these agents can be produced cheaply by aggressors and could have a larger impact on both military and civilian populations that other weapons of mass destruction. In the hands of a terrorist, these agents can cause great psychological harm and social disruption as well. Of the agents that can cause human disease, all except VEE and C. burnetii could be effectively transmitted through contaminated food and water.
Classical methods for identifying biological agents that cause human disease have been used for more than 100 years and are well established. These methods rely on agent cultivation, taking between 3 and 30 days, and require experienced personnel working in a well-equipped laboratory. In order to achieve agent identification with a high level of confidence, a combination of state-of-the-art immunological and nucleic acid analyses methods, as well as classical microbiological approaches, are needed to identify unknown biological agents. Methods based on the immunomagnetic-electrochemiluminescence assay for antigen detection are among the most sensitive.

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Sensitive and Rapid Identification of Biological Threat Agents

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Sensitive and Rapid Identification of Biological Threat Agents

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INTRODUCTION

The threat of biological warfare and bioterrorism has increased in the last two decades. Classical biological threats that were part of the now-defunct U.S. offensive program (terminated in 1969) included *Bacillus anthracis*, Botulinum toxin, *Francisella tularensis*, *Coxiella burnetii*, Venezuelan equine encephalitis (VEE), *Brucella suis* (brucellosis), Staphylococcal enterotoxin B (SEB), rice blast, rye stem rust, and wheat stem rust. Most of these agents can be produced cheaply by aggressors and could have a larger impact on both military and civilian populations than other weapons of mass destruction.[1] In the hands of a terrorist, these agents can cause great psychological harm and social disruption as well.[2] Of the agents that can cause human disease, all except VEE and *C. burnetii* could be effectively transmitted through contaminated food and water.[3]

Classical methods for identifying biological agents that cause human disease have been used for more than 100 years and are well established.[4] These methods rely on agent cultivation, taking between 3 and 30 days, and require experienced personnel working in a well-equipped laboratory. In order to achieve agent identification with a high level of confidence, a combination of state-of-the-art immunological and nucleic acid analyses methods, as well as classical microbiological approaches, are needed to identify unknown biological agents.[5] Methods based on the immunomagnetic-electrochemiluminescence assay for antigen detection and on polymerase chain reaction (PCR) gene amplification for nucleic acid detection are among the most sensitive.[6,7]

IMMUNOLOGICAL DETECTION PROTOCOLS

Electrochemiluminescence (ECL) is a process by which light is generated from a voltage-dependent, cyclic oxidation-reduction reaction of ruthenium heavy metal chelate. In the presence of tripropylamine (TPA), the redox reaction triggers the re-

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TABLE 1. ECL assay sensitivities for botulinum toxin type A, C fragment in various biological matrices.

Matrix	Sensitivity (tested)	Sensitivity (absolute)
PBS/Tween-20	1 pg/ml	50 fg
Skim milk	10 fg/ml	0.5 fg
Serum	10 fg/ml	0.5 fg
Urine	100 fg/ml	5 fg

lease of photons that are detected and quantified within a photomultiplier tube. Ruthenium is used in a low molecular weight, heme-like form that can be easily conjugated to any protein using standard *N*-hydroxysuccinimide (NHS) ester binding chemistries. In the conjugated form, ruthenium serves as an ideal tracer molecule because it has little or no influence on antibody-antigen interactions. Detection is facilitated in a homogeneous assay format using 2.8 μm magnetic beads that serve as the solid substrate for the detection reaction. The beads also provide both the basis for the separation of antibody-antigen complexes from potential non-specific reactants and the means to bring the specific reactants into the proximity of the electrode. A potential of only 2 volts across the electrode is required to initiate the ruthenium redox reaction. As a result of this low potential, only those ruthenium atoms within 30–50 nm of the electrode are detected, reducing the effect of non-specific reactants present in the assay suspension.

An ECL detection system consists of an analyzer and a personal computer with menu-driven ORIOS software. The system's strengths come from its speed, sensitivity, accuracy, and precision over a wide dynamic range. In a typical agent-detection assay, magnetic beads conjugated to a capture antibody and a ruthenium-conjugated detector antibody are added to unknown sample. The analyzer draws the processed sample from a vortexing carousel, captures and washes the magnetic beads, and quantifies the electrochemiluminescent signal. The current system is automatable, uses stable reagents, and is highly sensitive into the 0.1–1 pg/ml range. The total assay time with the current system is approximately 30 minutes, plus up to one minute per sample reading time. The next-generation ECL analyzers will include both handheld and high-throughput devices, making testing more accessible to caregivers and further decreasing the time required for testing and identification.

We have demonstrated the effectiveness of the ECL system for the detection of *Staphylococcus* enterotoxin B (SEB), ricin toxin, *Yersinia pestis* F1 antigen, *Bacillus anthracis* PA antigen, and Venezuelan equine encephalitis (VEE) virus. The technology could potentially be used with any biological agent and is limited only by the availability of high quality, high affinity antibodies or other ligands that can be used in the assay. This is of particular importance with regard to the detection of toxins per se, which are proteins and therefore not amenable to nucleic acid–based detection assays. In our hands, the technology is capable of detecting botulinum toxin at biologically significant concentrations (as low as 0.5 fg) for the first time. Representative results demonstrating the sensitivity of the botulinum toxin-detection assay are presented in FIGURE 1. As is the case with all agents tested, assay sensitivities vary between sample matrices (TABLE 1). Matrix-specific positive and negative control samples are used to establish standard curves and cutoff values. Using this ap-

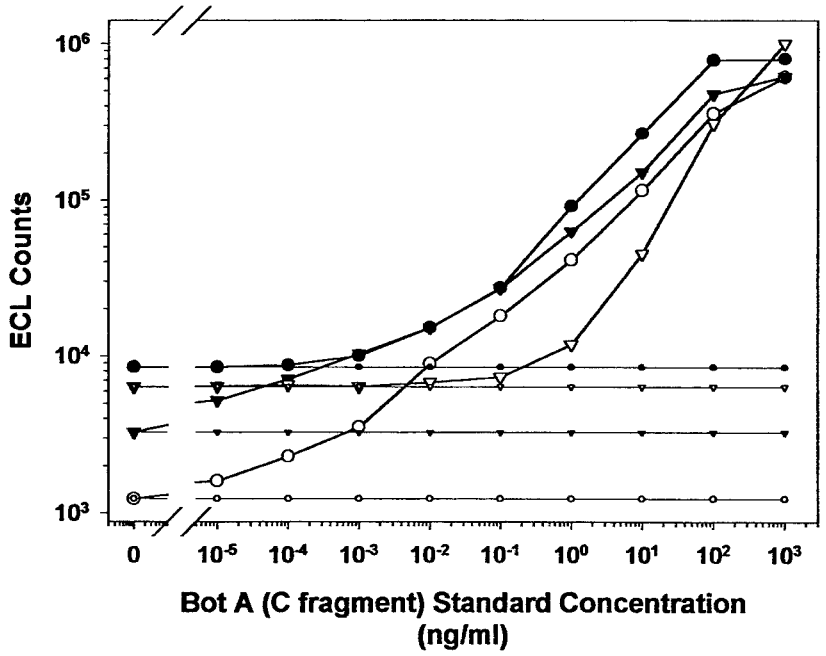


FIGURE 1. Standard curves generated from ECL analysis of various matrices spiked with Botulinum toxin type A, C fragment. *Closed circles* represent experiments completed in phosphate-buffered saline with 0.3% Tween-20; *open circles*, in skim milk; *closed triangles*, in serum; *open triangles*, in urine. Cutoff values are shown using narrow lines and small symbols, and were calculated from the mean of the results obtained from negative control samples, plus 2.5 standard deviations.

proach, any biological or environmental matrix can be tested and integrated into the testing platform. Using standard curves generated from test controls, the assays can be made fully quantifiable, increasing the utility of the technology even further. Such capabilities would be of great value when determining if a biological toxin is present in a food item or agricultural commodity at concentrations in excess of those expected from incidental contamination, i.e., a deliberate introduction.

The only major limitations of the ECL assay system revolve around the necessity for high quality reagents. Both detector and capture antibodies should ideally be highly specific and have high affinities for their respective antigens. All antibodies need to be highly purified to facilitate their efficient coupling to magnetic beads and ruthenium. However, once appropriate reagents have been identified and prepared, they are extremely stable, are easily lyophilized, and can be readily transported for field use. ECL-based agent identification is by far the most sensitive affinity-based diagnostic technology that we have tested to date. As the ECL hardware develops, this technology will prove itself to be an important component of an integrated approach to identification of potential biological weapons, both in the lab and in the field.

NUCLEIC ACID–BASED DETECTION PROTOCOLS

A variety of nucleic acid–based assays are currently available for detecting pathogenic microorganisms: the ligase chain reaction, the Qβ replicase-based system, the nucleic acid sequence–based amplification (NASBA) system, and PCR, among others.[8] Of these, PCR has been most extensively used for detecting DNA (deoxyribonucleic acid), and/or RNA (ribonucleic acid) unique to possible threat agents.

This technique involves the use of oligonucleotide primers (flanking a gene segment of interest), in conjunction with a heat-stable enzyme, e.g., *Taq* polymerase. By repeated cycling of the reaction through a range of temperatures permissible to the activity of the *Taq* polymerase, it is possible to synthesize large quantities of the desired gene segment to the exclusion of all other genes present in the sample.[7] The newly synthesized gene segments, or DNA molecules, are referred to as amplicons and can be analyzed and viewed by electrophoresis on agarose gels. When stained with the chemical dye ethidium bromide, amplicons are revealed as bands of fluorescing material in the gel. Because the investigator determines beforehand both the identity and length of the gene segment he or she is interested in amplifying, the size of the observed bands in the gel is indicative of an accurate and successful assay. It is possible to simultaneously assay a sample by PCR using as targets various gene segments, unique to a species or strain of microorganism. These can be used to both improve the detection limit and to determine if the organism in question contains particular genes associated with virulence or pathogenicity.

Because of its ability to amplify DNA from extremely small quantities of starting material, PCR offers DNA detection limits in the femtogram or even attogram range. Because it allows the investigator to design primers unique to gene segments of interest, PCR also offers a high level of specificity, with discrimination possible at the level of individual nucleotides, particularly when oligonucleotide probes (see below) are included in the assay. The PCR assay is also relatively quick to perform and analyze compared to culture and isolation techniques—depending on the need for sample preparation, results can be obtained in several hours.

PCR does have disadvantages. The assay requires pure and undegraded nucleic acids (templates) for optimum performance, making the removal of inhibitory substances, which are present in many clinical and environmental samples, a necessity.[9] Additionally, because of its exquisite sensitivity, contamination of PCR reactions with previously generated amplicons can result in false-positive assays. Avoiding contamination problems involves the use of specially designated laboratory equipment and handling procedures. Finally, successful use of PCR requires more involved training of personnel and the provision of properly equipped laboratories, with attendant requirements for larger budgets.

Sample Preparation

The minimum requirements for a sample preparation procedure for preparing PCR-amplifiable nucleic acid are (1) the release of the nucleic acid from the target organism in sufficient quantity and quality to support the *in vitro* amplification process and (2) the removal of PCR inhibitory contaminants arising from the sample matrix.[9] These requirements are influenced by a number of factors. Cell type is an important consideration. For example, it is more difficult to prepare DNA from *Ba-*

cillus anthracis spores than from vegetative cells under any circumstances. Another factor is the amount and type of PCR inhibitory contaminants present in the sample matrix. For example, it is more difficult to prepare DNA from *B. anthracis* vegetative cells from blood or soil samples than buffer samples. Furthermore, it is more difficult to prepare DNA from organic soils than from sandy soils. Finally, the type of nucleic acid being purified influences the sample preparation strategy. The ubiquity of ribonucleases (RNases) and their resistance to inactivation has to be taken into consideration when pathogens with RNA genomes or when RNA transcripts from an organism with a DNA genome are being targeted. Other features of a sample preparation procedure that are desirable and make it more likely that PCR will be routinely used in diagnostic laboratories are minimal use of hazardous materials, such as phenol, and maximum opportunity for automation to minimize the labor-intensive steps that hinder current sample preparation procedures.

There are a number of commercially available procedures for preparing nucleic acid for PCR. A successful approach for preparing DNA for PCR is based on variations of the Boom procedure.[10] In this method, organisms and their nucleic acid–protein complexes are lysed and disassociated with a chaotropic agent. Nucleic acid is then preferentially adsorbed to silica under high salt conditions, contaminants removed by selective washes, and DNA preferentially eluted with a low salt buffer. Qiagen, Inc. (Chatsworth, CA) produces a variation of this procedure in two forms: the QIAamp® Tissue Kit and the QIAamp® Blood Kit. The Qiagen procedures incorporate a protease digestion step to enhance the chaotropic salt–mediated lysis of cells. These two procedures are identical except for the protease digestion step. In the tissue kit procedure, there is a 60-min incubation at 55°C with proteinase K, before a 10-minute incubation at 70°C in the chaotropic salt–containing lysis buffer. The blood kit procedure combines the protease digestion step with the chaotropic disruption step during the 10-min incubation at 70°C. Instead of proteinase K, the procedure uses Qiagen protease, a Qiagen product optimized specifically for preparing DNA from blood. We found that the tissue kit procedure was better for preparing spore samples in buffer, i.e., the endpoint detection limit for it was greater than for the blood kit, 4×10^3 colony-forming units (cfu)/40 µl sample versus 4×10^4 cfu/40 µl sample. We speculated that the proteinase K digestion step was more efficient for preparing spores for DNA extraction by this method. However, we also found that the most sensitive method for preparing spores for DNA extraction was to germinate them for 60 minutes. After this treatment the spores behaved like vegetative cells and we were able to detect $4 \times 10^1 - 4 \times 10^2$ cfu/40 µl sample.

Although the procedure is labor-intensive, with multiple centrifugation steps, and is therefore not readily adaptable to automation, it is very reliable and sensitive. It is also independent of the sample matrix. We found we could prepare *B. anthracis* vegetative cells with equal endpoint detection limits, from whole blood, plasma, or serum (FIG. 2). Preliminary experiments with *Yersinia pestis*, the etiologic agent of plague, and *Brucella abortis*, the etiologic agent of brucellosis, indicate that the Qiagen procedures will also be effective in preparing these agents for PCR.

In an effort to take advantage of the efficacy of this technology and to minimize its labor-intensive shortcomings, we are evaluating the Autolyser®, an instrument developed by XOHOX Research Institute (Menlo Park, CA). It fully automates this process. The Autolyser can be transported in a suitcase, complete with reagents and

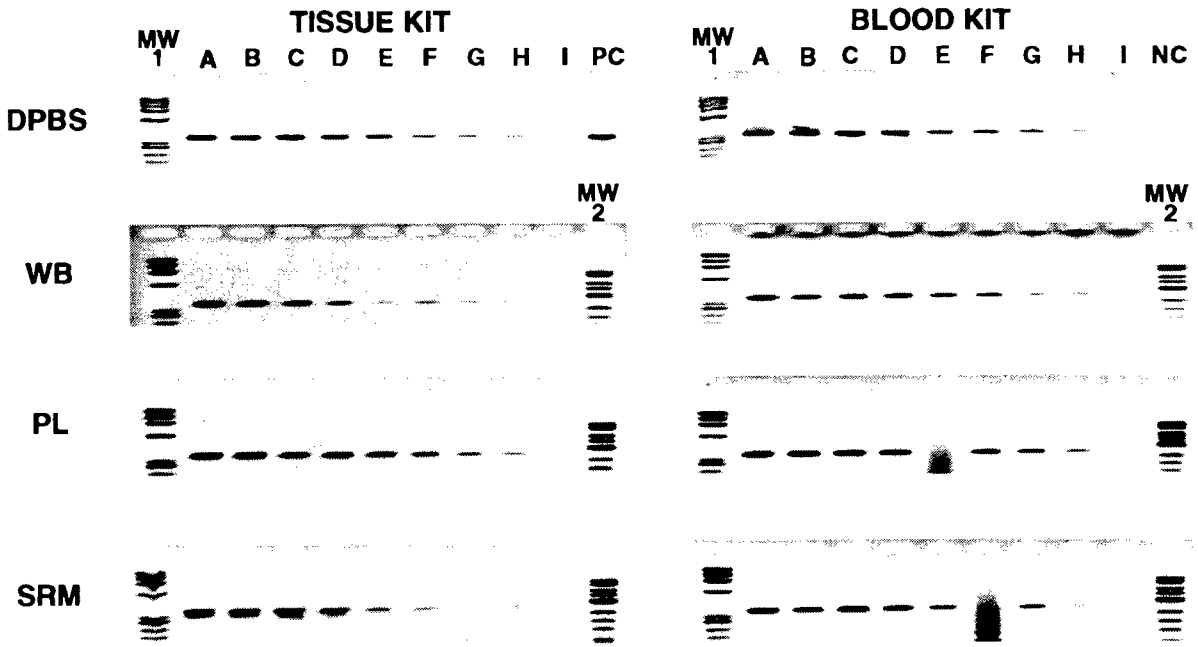


FIGURE 2. Comparison of tissue kit and blood kit procedures for preparing samples diluted in Dulbecco's phosphate-buffered saline, whole blood, plasma, and serum. Serial dilutions of γ-irradiated *Bacillus anthracis* vegetative cells were prepared in Dulbecco's phosphate-buffered saline (DPBS), whole blood (WB), plasma (PL), and serum (SRM) and processed by either the tissue kit or blood kit procedure. Colony-forming units/40 µl sample: (A) 5.0×10^4; (B) 2.5×10^4; (C) 1.2×10^4; (D) 6.2×10^3; (E) 3.1×10^3; (F) 1.5×10^3; (G) 7.8×10^2; (H) 3.9×10^2. Sample I is a no sample, diluent control. PC = PCR positive control; NC = PCR negative, no template control. MW1 = ΦX174 *Hae*III molecular weight markers; MW 2 = ΦX174 *Hin*fI molecular weight markers.

a laptop computer with menu-driven software. After a sample is introduced into a disposable extractor device, the instrument automatically dispenses lysis, wash, and elution reagents according to user-modifiable software instructions. Purified nucleic acid is deposited into a sterile vial approximately 15 minutes after sample introduction. The procedure requires no additional laboratory equipment and requires minimal training to operate. With this instrument we have successfully prepared PCR-amplifiable DNA from *B. anthracis* spores and vegetative cells.[11] Currently, the endpoint detection limit of PCR performed on Autolyser-extracted DNA is not as great as for DNA extracted with Qiagen systems; however, the Autolyser's ease of use justifies further development.

We have also evaluated two relatively new technologies based on modifications of Schleicher and Schuell 903 paper: FTA® paper, manufactured by Flinders Technologies, Inc. (Adelaide, South Australia), and distributed by Fitzco, Co. (Maple Plain, MN) and Life Technologies, Inc. (Bethesda, MD); and IsoCode® Cards, manufactured and distributed by Schleicher and Schuell (Keene, NH). Both procedures are optimized for preparing DNA from whole blood and employ proprietary chemistries that remove or inactivate blood-specific PCR inhibitors. In the FTA procedure, a sample is spotted onto the paper and dried. A 3-mm punch from the paper is processed through a series of washes with a specific FTA purification reagent, Tris EDTA buffer with 0.1 mM EDTA, and ethanol, to remove inhibitors. The processed punch with the captured DNA is directly used in the PCR reaction. With the IsoCode procedure, the sample is also spotted, dried, and punched, but is subjected to a single H_2O wash. After the wash the DNA is eluted from the punch by heating to 95–100°C for 30–45 minutes. The eluate and paper both contain amplifiable DNA. Experiments are in progress to determine whether we can eliminate the elution step without adversely affecting sensitivity. Preliminary experiments indicated that both the FTA and IsoCode procedures were approximately equally sensitive for preparing DNA from *B. anthracis* vegetative cell from buffer and whole blood samples. However, in our hands, FTA paper was significantly less efficient in preparing *B. anthracis* DNA from either plasma or serum. Because of this deficiency and because of the relative simplicity of the IsoCode procedure—one water wash, versus multiple washes with different reagents—we decided to further evaluate the IsoCode paper procedure as a means for preparing DNA for PCR. We found the procedure to be very effective, enabling us to detect 2×10^2 cfu/20 µl sample of germinated *B. anthracis* spores or vegetative cells. Like the Qiagen procedures, detection was independent of sample matrix (FIG. 3).

For RNA targets (i.e., viruses), one of the most effective methods for preparing RNA for amplification by the reverse transcriptase (RT)-PCR procedure is a variation of the Chomczynski and Sacchi procedure[12] in which cells are lysed and protein–nucleic acid complexes dissociated by a chaotropic agent, such as guanidinium isothiocyanate (GITC). The RNA is then extracted with acidic phenol–chloroform–isoamyl alcohol and concentrated by alcohol precipitation. In addition to being an effective agent for lysing cells and disassociating the RNA–protein complexes, GITC effectively inactivates RNases, satisfying an essential requirement for preparing RNA targets. There are a number of commercially available systems that employ procedures based on this technology. We have tested the RNAgents™ System from Promega, Inc., (Madison, WI) and a variation of the TRIzol procedure (Life Tech-

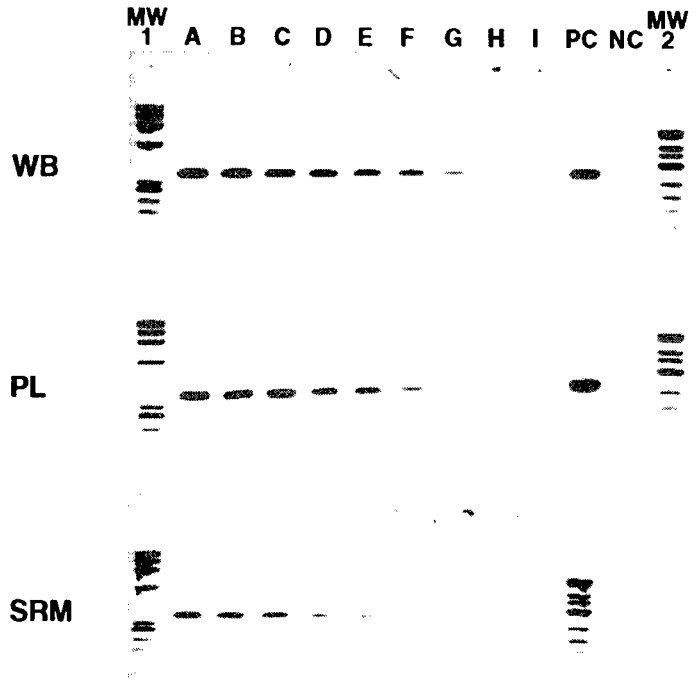
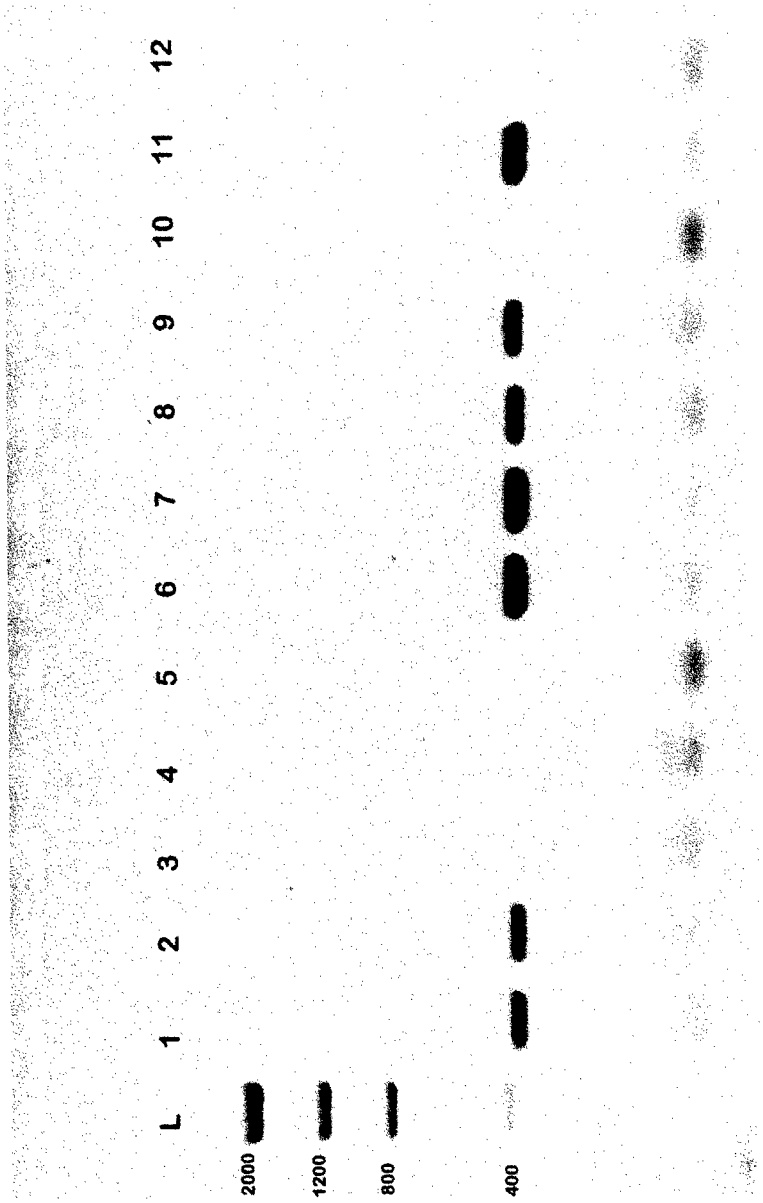


FIGURE 3. Comparison of IsoCode® paper for preparing vegetative cells diluted in whole blood, plasma, or serum. Gamma-irradiated *Bacillus anthracis* vegetative cells were diluted in whole blood (WB), plasma (PL), and serum (SRM) and prepared by the IsoCode procedure. Colony-forming units/20 µl sample: (A) 2.0×10^5; (B) 1.0×10^5; (C) 5.0×10^4; (D) 2.5×10^4; (E) 1.2×10^4; (F) 6.2×10^3; (G) 3.1×10^3; and (H) 1.5×10^3. I is a no sample, diluent control. PC = positive PCR template control; NC = negative PCR no template control. MW1 = ΦX174 *Hae*III molecular weight markers; MW2 = ΦX174 *Hin*fI molecular weight markers.

nologies, Inc., Bethesda, MD) in which the GITC and phenol are combined into a single reagent instead of two separate ones.[13] This procedure is very effective for preparing RNA from RNA-genome viruses, such as Rift Valley fever virus (RVF), or Venezuelan equine encephalitis virus (VEE), enabling us to detect between 200–1,500 plaque-forming units (pfu)/100 µl, depending on experimental conditions. However, the procedure is labor-intensive, not readily adaptable to automation, and uses hazardous chemicals that require special handling and disposal.

Another procedure we have investigated for isolating nucleic acid is immunomagnetic-separation (IMS). In this procedure, paramagnetic beads are derivitized with a specific antibody. These derivitized beads are used to capture a select organism from the sample milieu in much the same way as capture antibodies are used to immobilize specific antigenic targets onto microtiter plates for ELISA procedures. The advantages of this procedure are that it can effectively remove the target organism from the sample milieu, thus eliminating potential PCR inhibitory contaminants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in the process. At the same time, because of the high surface area to volume ratio of the beads, this procedure can also concentrate the analyte. Moreover, this procedure has the potential for automation. We have developed IMS procedures for VEE and have found that this technique increased the sensitivity of subsequent RT-PCR, enabling us to detect 25–100 pfu/100 µl sample, equivalent to an eightfold increase in detection limit over the GITC–acidic phenol extraction procedure. We have also developed an IMS procedure for two bacteria, *Yersinia pestis* and *Brucella abortis*, and found that DNA isolated with this method yielded PCR detection limits at least as sensitive as DNA isolated with the Qiagen procedures. The IMS extraction procedure had the added advantages of being able to concentrate dilute targets from larger volumes and more amenable to automated operation.

As an example of how rapid sample preparation methods and PCR might be applied to the investigation of a food item or an agricultural material suspected of harboring a biowarfare agent, we performed an experiment in which either a 1-ml or 10-µl aliquot of whole milk was spiked with serial log dilutions of *Bacillus anthracis* Ames vegetative cells. We used these two volumes for our spiking experiment to determine if the inhibitory action of lipids or proteins present in the milk could be removed using the Isocode sample preparation method. After addition of the *B. anthracis*, the milk was vortexed to disperse the bacteria thoroughly, and a 10–µl portion was spotted onto Isocode disks (about 6 mm in diameter) and processed. A 10-µl sample of the eluate was used as template for *B. anthracis* protective antigen gene amplification.

Results are shown in FIGURE 4. When 10-µl aliquots from the 1-ml spiked milk samples were assayed (lanes 1–5), the detection limit was approximately 1,000 cfu per aliquot (Lane 2). When smaller portions (10 µl) of milk were spiked with *B. anthracis*, the detection limit of the assay was approximately 100 cfu per aliquot (Lane 9). This assay indicates that it is possible to detect *B. anthracis* in milk at concentrations that may be encountered in a possible bioterrorism scenario using an inexpensive, simple sample preparation technique. It should be noted that, as with other PCR assays, the detection limit can undoubtedly be improved by the use of either a nested PCR assay or a PCR-enzyme immunoassay (see below); but these techniques can add several hours to the overall assay time.

PCR-ENZYME IMMUNOASSAY

The PCR-enzyme immunoassay (PCR-EIA) is a relatively recent development in PCR technology. The basic assay reagents are commercially available (Boerhinger Mannheim). The assay utilizes the incorporation of an altered nucleotide, digoxige-

FIGURE 4. Detection of *Bacillus anthracis* Ames vegetative cells in whole milk. One-ml and 10-µl volumes of milk were spiked with a dilution series of colony-forming units (cfu), and 10-µl aliquots spotted onto Isocode® paper and processed for PCR with the BAN 23 primer set. Lane L: DNA mass ladder with sizes of the rungs indicated. Lanes 1–4: aliquots from 1 ml milk spiked with 2×10^5, 1×10^5, 1×10^4, and 1×10^3 cfu, respectively. Lane 5: unspiked milk control. Lanes 6–9: aliquots from 10 µl milk spiked with 2×10^4, 1×10^4, 1×10^3, and 1×10^2 cfu, respectively. Lane 10: unspiked milk control. Lane 11: *B. anthracis*–positive control. Lane 12: no template control.

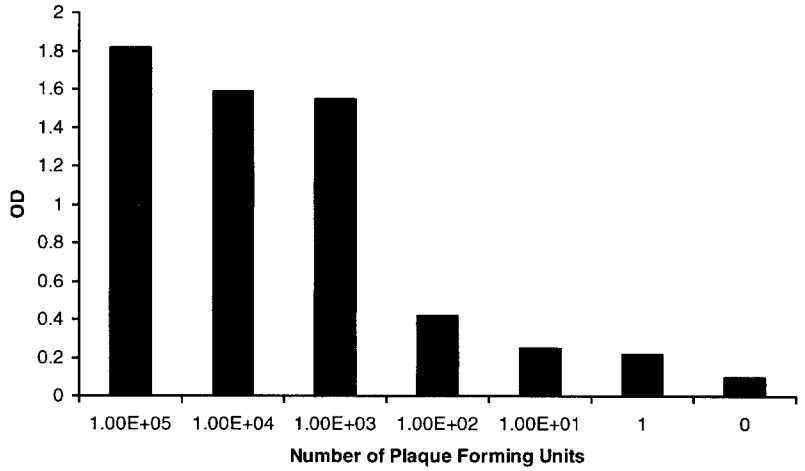

FIGURE 5. Detection limit of PCR-EIA for Venezuelan equine encephalitis virus. The optical density units are plotted on the *y* axis, and the number of plaque-forming units of VEE are plotted on the *x* axis.

nin-11-deoxyuridine triphosphate (d11-dUTP), into amplicons. These are in turn subjected to hybridization with a biotin-labeled oligonucleotide probe whose sequence is determined by the investigator. Probe bound to target regions on the amplicons is visualized by a colorimetric enzymatic reaction, mediated by an antibody and a colorimetric substrate. The assay is performed using a microtiter plate format, with positive reactions indicated by color change, in proportion to the quantity of template in the samples. A successful assay can detect femtogram quantities of pathogen DNA. The assay allows many samples (up to 96, including controls) to be processed at once. The use of a biotin-labeled, oligonucleotide probe improves specificity compared to conventional PCR. The drawbacks of PCR-EIA are that it requires between 2–6 h to complete and involves multiple pipetting steps. We have developed PCR-EIA assays for the detection of bacterial and viral threat agents. An example is depicted in FIGURE 5; here, the PCR-EIA allows detection of as little as 1 pfu of VEE virus.

Fluorogenic Probe-Based PCR

These techniques incorporate fluorescent dye molecules either as stains for the amplicons themselves or attached to oligonucleotide probes, which then hybridize to the amplicons, into the PCR reaction. The advantage of using fluorogenic markers in PCR is that with the appropriate detection platforms, the accumulation of amplicons can be monitored in "real time," i.e., as the reaction(s) progress. Data can be presented as amplification plots on a graph, obviating the need for gel electrophoresis to determine if a given sample is positive. A number of different assay systems and platforms are commercially available. One such system is TaqMan® 5′ nuclease assay (5NA).[14]

The TaqMan 5NA (Perkin Elmer/Applied Biosystems) utilizes an oligonucleotide probe, whose sequence is selected by the investigator, double-labeled with fluorescent reporter and quencher dye molecules. As amplicons accumulate during the course of the reaction, the probe will hybridize to any target sequence present on the amplicons. When exposed to pulses of intense light (e.g., a laser), the fluorescent dyes located on the probe will respond with characteristic emission spectra. These spectra can be monitored by an appropriate platform and data collated and presented to the user as amplification plots.[15] Because different probes can be labeled with different dye molecules, it is possible to simultaneously assay the same sample for the presence of different target sequences (i.e., "multiplexing"). In practical terms, this would allow the same sample to be assayed for the presence of several different threat agents in a one-tube reaction.

The main advantage offered by the 5NA is that with real-time monitoring, results can be obtained much quicker than with conventional PCR or PCR-EIA. It is also possible to design probes that allow discrimination between single nucleotide differences in gene sequences. As demonstrated below, this can be of importance in differentiating between pathogenic and nonpathogenic microorganisms.

The use of probe-based, nucleic acid amplification protocols also provides investigators with the ability to identify "artificially created" pathogens, which, unfortunately, are feasible using modern molecular biology techniques. An experienced molecular biologist could insert genes coding for toxins, virulence factors, or antibiotic-resistance proteins into bacterial and viral threat agents.

To address the possibility of encountering pathogens that have been "fortified" with virulence genes from other unallied microorganisms, USAMRIID's diagnostic research is focusing on rapid detection and identification of a number of known virulence genes for a variety of pathogens. It is feasible, for example, to perform molecular tests on a given bacterial or viral isolate for the presence of introduced toxin genes, which would not normally be detected in conventional assays. As research into these detection capabilities proceeds, it is hoped that it will provide authorities with the ability to rapidly determine if a given isolate is deliberately altered at the genetic level to enhance its use as a biological weapon.

We have functional TaqMan 5NA for a variety of bacterial and viral threat agents: *B. anthracis*, *Y. pestis*, *Brucella* spp., *F. tularensis*, *Staphylococcus* enterotoxin B gene, Venezuelan equine encephalitis virus, and a number of orthopoxvirus species. The *Y. pestis* assay has been successfully used to detect bacterial DNA in oropharyngeal swabs taken from monkeys infected with aerosolized formulations of *Y. pestis* and in artificially infected vector fleas.[16] Our orthopoxvirus assays allow differentiation of strains at the single-nucleotide level.[17,18] Because of the high degree of sequence homology among the orthopoxviruses, this capability is vital to determine if a virus isolate is pathogenic for humans or is an animal virus whose presence in the environment is otherwise unremarkable.

An example of the use of rapid, probe-based PCR methods to identify material from an actual incident involving a potential threat agent is provided in FIGURE 6. A law enforcement agency provided the Special Pathogens Department at the Diagnostic Systems Division with a bacterial preparation suspected of containing *B. anthracis*. Bacterial colonies cultured from the confiscated material were assayed by 5NA, using probes to the protective and capsular antigens of *B. anthracis*. In FIGURE 6, the

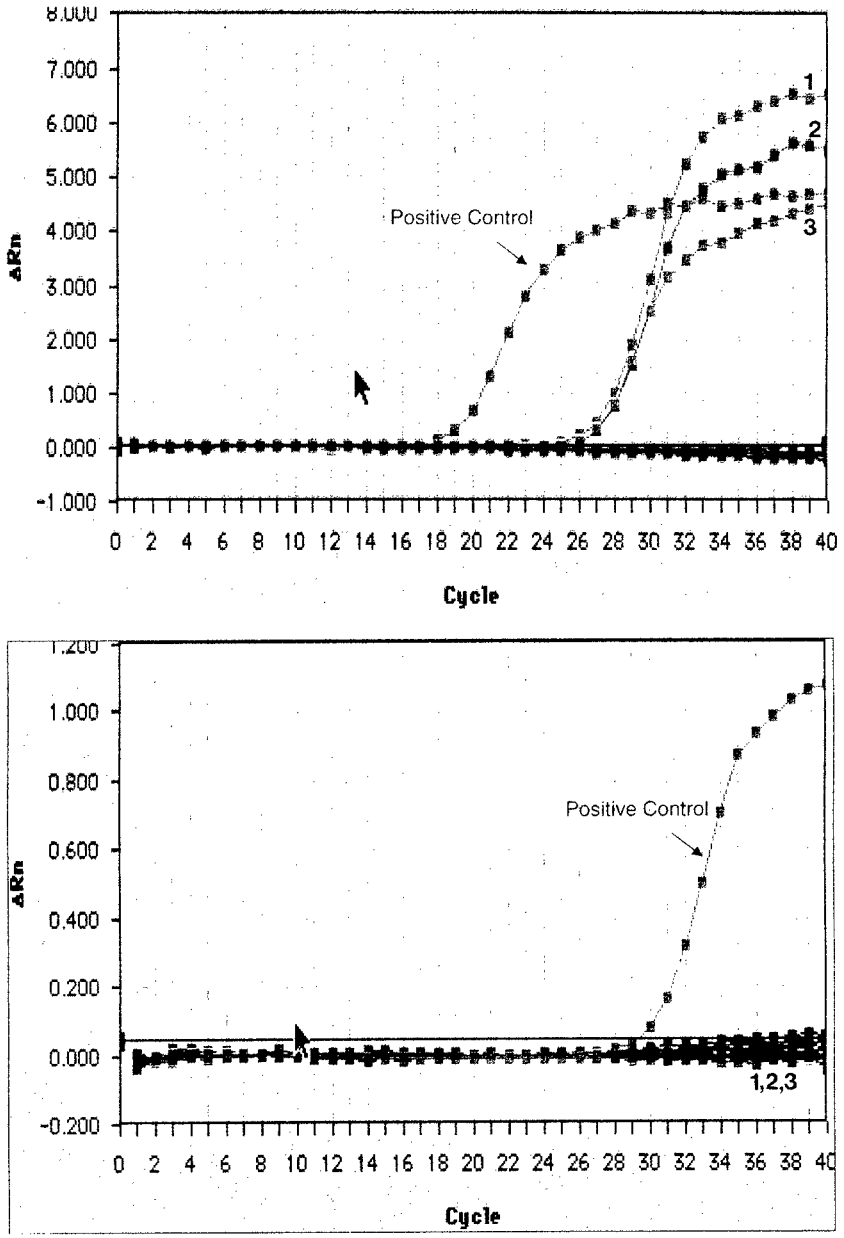

FIGURE 6. Fluorogenic probe-based PCR for *Bacillus anthracis*. The ΔRn values are plotted on the *y* axis, and the PCR cycle numbers are plotted on the *x* axis. The plot for the positive control sample is indicated; the plots for the unknown samples ($N = 3$) are designated by number. Amplification plots located below the threshold line ($\Delta Rn = 0$) are considered negative, and include no template controls. (A, top) The unknown samples were assayed with primers and probe for the *B. anthracis* protective antigen gene. (B, bottom) The same samples were assayed with primers and probe for the *B. anthracis* capsular antigen gene.

amplification curves for each sample assayed are plotted on a graph. The ΔRn values (*y* axis) are indicative of probe activity; samples with plots below the threshold line (ΔRn = 0) are considered negative. The samples were positive when assayed with primers and probe for the protective antigen (FIG. 6A). However, when assayed with primers and probe for the capsular antigen gene, a loci associated with virulent strains of anthrax, the samples were negative (FIG. 6B). The conclusion from this and other data was that the sample was an avirulent strain of *B. anthracis* conventionally used in vaccine preparations. The assay demonstrated that the sample lacked the capsular antigen gene, which is associated with virulent strains of *B. anthracis*. Results such as these, which were obtained within 2 h of beginning the 5NA, can have obvious implications in determining safety measures with regard to exposure (accidental or otherwise) to such a bacterial preparation. Information from 5NA analyses of confiscated material can also be a useful component of forensic investigations.

FUTURE DIRECTIONS FOR NUCLEIC ACID–BASED DETECTION ASSAYS

A significant disadvantage of fluorogenic probe assays is the requirement for expensive, bulky platforms for real-time monitoring of the reactions. This lack of portability can hamper the ability of real-time PCR to be carried out in field situations. Accordingly, the Department of Defense, in conjunction with investigators at the Department of Energy's Lawrence Livermore National Laboratories, has sponsored research into the development of miniature analytical thermal cyclers. One such platform is the MATCI (miniature analytical thermal cycler instrument) manufactured by Lawrence Livermore National Laboratory.[18,19] The MATCI utilizes silicon wafers to mediate the heating and cooling of the reaction tube, in conjunction with light-emitting diode (LED) stimulation of fluorogenic probes present in the PCR tube.[19] The entire apparatus is suitcase-size, portable, and offers "true" real-time monitoring of probe activity (i.e., the amplification plots onscreen are continually updated cycle by cycle). The MATCI has been successfully used to detect, and differentiate between, orthopox virus species.[18]

An improved version of this platform is designated the Automatic Nucleic Acid Analyzer (ANAA) and features ten independently programmable heating blocks.[20,21] When *Bacillus subtilis* spores (used to simulate *B. anthracis* spores) were assayed by fluorogenic probe–based PCR in this instrument, positive signals were viewed in 18–26 minutes.[20] A handheld platform offering equivalent real-time monitoring capabilities, with four blocks, is in the prototype stage and may be affordable to various investigators and laboratories by the year 2000 (P. Belgrader, Lawrence Livermore National Laboratories, personal communication).

Other Department of Energy–sponsored biological weapon detection technologies include the MiniFlo cytometer, which uses immunofluorescence-based sensors to detect both pathogens and toxins. In field exercises held in 1996 at Dugway, Utah, 1,600 analyses on 400 samples yielded an 87% positivity rate, with less than a 0.5% false positive rate.[21]

Researchers at Cepheid, a private firm in Sunnyvale, CA, are also constructing portable thermal cyclers with real-time capabilities with the goal of marketing hand-

held platforms offering combined microfluidics-based sample preparation, thermal cycling, and data presentation functions. The reaction chamber heating and optics functions would be mediated via the use of a specially designed ceramic chip ("I-CORE"). The Smartcycler™, offering real-time, fluorogenic probe-based PCR, is expected to be marketed within the year. Ultimately, a Cepheid system would be capable of accommodating sample volumes as large as 1–3 ml. This is of importance when few pathogens are expected to be present in the sample. The company plans to develop a briefcase-size thermal cycler unit with integrated sample processing; the instrument would be available within the next several years and priced to be affordable to a variety of end users.[22,23]

We have demonstrated that our orthopoxvirus 5NA is readily adaptable for use on these novel platforms, performing as well as or better than commercial instruments.[18] Ongoing research in our laboratory has indicated that with such platforms a nucleic acid–based detection procedure—from sample preparation, amplification, and determination of results—can take less than one hour.[18]

APPLYING ADVANCED DIAGNOSTIC TECHNIQUES IN BIOTERRORISM SCENARIOS

How feasible is it to move these sophisticated laboratory instruments and assays from the bench to the field, from an essentially reactive to a proactive role? The U.S. Army has been deploying rapid diagnostics capabilities in the field since the 1980s. More recently, during Operation Vigilant Warrior (1994), we demonstrated for the first time that advanced molecular diagnostics using PCR can be performed under field conditions for identification of disease agents. Subsequently, the 520th Theater Area Medical Laboratory (TAML, currently commanded by Colonel William Chambers, and stationed at Aberdeen Proving Ground in Maryland) was activated. TAML is a self-contained laboratory that can be airlifted to overseas locations. It provides theater-level laboratory support for preventive medicine activities, using a variety of sophisticated diagnostic assays. The laboratory is staffed by enlisted personnel and has demonstrated an ability to conduct diagnostic analyses with a high degree of accuracy and reproducibility over several months under field conditions. For example, the 520th TAML was recently deployed to an overseas location in support of U.S. Army personnel. Despite difficult environmental conditions (high ambient temperatures that made it necessary to perform the bulk of the assays after sunset, sand infiltration of equipment, etc.) the laboratory conducted numerous detection assays each day. Results obtained from the TAML assays were confirmed at USAMRIID, indicating that TAML operations represent a highly successful transfer of technology from the research laboratory to the field (T. Cao and F. Knauert, unpublished data). Therefore, it is feasible to envision a civilian counterpart with the same capabilities as the 520th TAML.

How would rapid detection techniques such as the ones described above be used in the event of intentional contamination of food or water with pathogenic agents? Because such incidents are rare, it is necessary to extrapolate from what we do know occurred in the United States in The Dalles, Oregon, in 1984. There, a large outbreak of salmonellosis among patrons and workers at 10 restaurants was ultimately as-

cribed to deliberate contamination of salad bar foods by members of the Bhagwan Shree Rajneesh religious cult. In addition to the restaurants, cult members also contaminated produce at a grocery store and intended to contaminate the municipal water supply as well. Evidently the cult had hoped to cause enough widespread illness to significantly lower voter turnout on a land-use balloting issue that would have hampered the cult's activities. It took more than a year for investigators to accumulate enough evidence to identify the cult as the source of the outbreak. This was aided by seizure of a *Salmonella* culture from the Rajneesh Medical Center. A panel of biochemical and genetic assays indicated that it was identical to the strain isolated from outbreak patients.[24] At the time of the outbreak and investigation (1984–1985), PCR-based techniques were not yet invented

The Oregon outbreak ultimately involved at least 751 cases, with 45 people ill enough to be admitted to the hospital. It is troubling to realize that while the cult had selected a relatively benign pathogen, it was easily obtained from a commercial microbiological specimen provider; it was cultured in the Rajneesh laboratory without the need for special facilities and equipment; and it was spread simply and easily throughout the community without attracting any undue suspicion. Soon after the epidemiologic investigations began, the authorities did have suspicions that the outbreak was intentional, but for various reasons, this hypothesis was not immediately pursued.[24]

More recently, in 1996, in a Texas medical center laboratory, 12 workers were diagnosed with *Shigella dysenteriae* infection. The source of the infection was traced to pastries anonymously left in a common break room. Because the facility routinely performed microbiological assays, bacterial stocks and culturing equipment were kept on the premises. Investigators examined stool sample isolates and bacterial stocks from the lab storage freezer. Using pulse-field gel electrophoresis, a technique that allows comparative analysis of chromosomal DNA profiles of different bacterial strains, they determined that the *S. dysenteriae* strains from the freezer and stool samples were identical. The investigators concluded that the pastries had been deliberately contaminated and a criminal investigation was begun.[25]

In both incidents, the realization that deliberate contamination of food items was the source of infection happened only after the outbreaks had occurred. This underscores how difficult it can be to prevent or ameliorate illnesses caused by exposure to covertly contaminated food or water sources.[26] The use of gastrointestinal pathogens, which are relatively easy to acquire, can confound attempts to determine if the outbreak was triggered by natural or intentional activities. For example, it would require an intensive effort to distinguish between an outbreak caused by naturally present botulinum toxin and one caused by a deliberately introduced formulation.

Alternatively, since biowarfare agents such as anthrax or encephalitis viruses would constitute more exotic pathogens, for many health departments confronted with unusual symptoms among a population exposed to contaminated food or water, arriving at a timely and accurate diagnosis would be difficult unless select medical personnel had previous experience with illness caused by these agents. The symptoms of gastrointestinal anthrax, an extremely rare disease in nature, are similar to those of most gastrointestinal tract infections, with fever, diarrhea, nausea, and vomiting predominating before the infection progresses to acute abdominal pain, at which time the infection is most serious and mortality rates can approach 50%.[27]

The utility of rapid detection assays would therefore seem to address two areas: (1) prophylactic monitoring of food or water suspected of being the target of a bioterrorist attack and (2) serving as "first use" diagnostics when an otherwise routine outbreak of gastrointestinal illness shows evidence of being something else entirely. (We wish to stress that rapid detection techniques would certainly not be used alone in any effort, but would be used in conjunction with more traditional techniques, such as culturing of suspected agents, animal assays, as well as highly specific immunodiagnostic assays, such as ECL).

With regard to monitoring of food or water sources suspected of having been contaminated with biological warfare agents, we feel that the rapid sample preparation techniques and real-time diagnostic assays developed at USAMRIID would allow authorities to perform the quickest and most accurate tests to determine if the threat is real. We envision the on-site use of multiplex, probe-based PCR to simultaneously assay samples for the presence of a number of possible infectious agents, in conjunction with field-based immunoassays for confirmation of PCR procedures and detection of various toxins. Results of these assays would allow authorities to take appropriate countermeasures, such as securing the food or water source to prohibit its use, and also constitute evidence for criminal prosecution.

In the event of an outbreak of gastrointestinal illness suspected to have been caused by the deliberate introduction of biological warfare agents into food or water items, the use of real-time, rapid diagnostics would be of importance in establishing early diagnoses for optimum treatment and protective measures. Because most public health laboratories lack the reagents and expertise required to diagnose disease caused by agents such as poxviruses and anthrax or toxins such as ricin, timely access to a "package" of rapid detection instruments and assays would be critical.

In addition to the advantages described above, it may be helpful to think of rapid detection capabilities as contributing to a well-formulated and effective policy of deterrence of biological warfare/terrorism.[28] In conjunction with other countermeasures, such as drug prophylaxis, immunizations, protective clothing, and shelters, rapid diagnostics can help to minimize casualties and serve as a signal to potential adversaries that the United States is becoming less vulnerable to these attacks.

In conclusion, we are confident that rapid detection assays can provide crucial information to personnel confronted with an incident of suspected bioterrorism. Alternatively, these assays can be utilized in a proactive mode to monitor vulnerable commodities for safety and hygiene. Such assays could be conducted within hours of receiving samples, providing results more rapidly than techniques currently available in most diagnostic laboratories. The sensitivity and accuracy of rapid diagnostic techniques, augmented by traditional assays, would provide the abovementioned personnel with a valuable resource for use against biological warfare or terrorism aimed at the nation's food and water resources.

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